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EFFECT OF THE GLUCOSE CONTENT IN THE NUTRIENT MEDIUM ON THE DEVELOPMENT OF TISSUE CULTURE

- Poland -

/Following is a translation of an article by Izabela Polna and Halina Leszczynska, Institute of Virology of the State Institute of Hygiene, Warsaw, in the Polish-language periodical Medycyna Doswiadczalna i Mikrobiologia (Experimental Medicine and Microbiology), No. 14, 1962, pp 365-377/.

The first reports concerning the effect of the individual components of the nutrient medium on the in vitro growth of tissue culture date from the 1930's.

Willmer (21), Parker (18) and Eagle (9-10), in studying the chemical changes occurring in the nutrient medium from the viewpoint of the relationship between the metabolism of developing tissue and its growth (in its mass and on the surface), addressed their greatest attention to the role of glucose.

Willmer (21), in describing the development of the tissue of the viscera of the chicken embryo in different solutions of various salts, devoted special attention to the effect of glucose on the growth of cells. He observed that the total number of multiplying cells increased together with the growth of the glucose content in the nutrient medium. The upper limit of concentration of the glucose in the nutrient medium at which this simple dependence occurs is 1,000 mcg%; any higher concentration appears to exert a certain toxic action.

A similar role of glucose in the multiplication of cells was also noted by Demuth and Ebeling (8, 11). The latter found that the growth of fibroblastic cells becomes intensified

when the level of the glucose rises from 200 mcg% to 1,150 mcg% -- at higher concentrations of this sugar tissue growth is subject to retardation.

Krontowski (13) showed that the tissue can consume in 48 hours more glucose than its own mass.

Morgan and Kutsky (15) studied the effect of glucose on the growth of chick liver explantations. They found that tissue devoid of a source of glucose does grow, but its growth is weaker than that of tissue cultures in a medium with a normal quantity of glucose, i.e., 0.1%. Morgan and Kutsky observed, however, that the growth of explantations occured only in those cases where this tissue culture was in an oxygen medium, whereas growth did not take place at all when oxygen was replaced by nitrogen.

Also studied was the ability /of tissues/ to assimilate sugars depending upon the kind of tissue. Nelson and Spratt (16-17) found that the sugar requirements of blastoderms were not the same as those of fibroblasts. Blastoderms in a medium without glucose are subject to degeneration, and no other sugar can replace it. Fibroblasts, on the other hand, can grow in a sugarless medium. Spratt observed that the role of sugars differs in different periods of growth of the chick blastoderms. He found that many differences occur in food requirements. These differences correspond to the period of development, viz. in the phases of morphogenesis, differentiation and growth.

These food requirements may also differ according to the kind of tissue, even if the various tissues come from the same organism. For example, heart tissue can develop on various sugars, but brain tissue develop only on glucose.

Observations of the process of assimilation of glucose by the cell were made by Gothoskar, Raina and Ramakrishnan (12). They noted that fragments of chicken and rat embryo hears incubated for 24 days have three glucose-intake periods: the first period, when glucose consumption is growing; the second period, when the quantity of glucose consumed is constant; and the third period -- the final one -- when there is a drop in glucose consumption. The differences in glucose consumption in the last two stages are insignificant.

It has been observed that glucose consumption depends upon the age of the organism from which the tissue was isolated.

Thus, the tissues of the chick embryo consume larger quantities of glucose than the tissues of the adult chicken.

In becoming better acquainted with the mechanism of glucose assimilation, the object of our work was to ascertain the following:

1) Consumption of glucose by monkey kidney cells during a week's observation and the correlation occurring between the growth of tissues and the consumption of glucose;

2) Can the fluid from atop a seven-day culture be used again to start a new culture, after adding to it only 2% calf serum without supplementing the glucose content (regenerated fluid)?;

3) How does the growth of monkey kidney tissue proceed in a medium devoid of a source of sugar?;

4) What is the multiplication of poliomyelitis virus depending upon the glucose concentration in the nutrient fluid?

MATERIALS AND METHODS

A. The following were used for the experiments: 1) Epithelial cells of monkey kidneys (species Mocaca Mulata /rhesus monkey, or Bengal mocaque/ and species Mocaca irus /crab-eating mocaque/, both species of the sub-family Cercopithecinae of the family of tailed monkeys). 2) Calf serum (always from one take, i.e., of the same series); dialyzed calf serum, likewise from the same series. The serum was dialyzed in PBS for 48 hours at room temperature, with agitation 3) The following nutrient media: a) normal medium -- Hanks fluid containing 0.1% glucose + 0.5% hydrolyte of lactoalbumin + 2% calf serum (undialyzed); b) Hanks fluid without glucose + 0.5 hydrolyte of lactoalbumin + 2% calf serum; c) regenerated fluid (by regenerated fluid we mean a fluid such as we have described in a), above, taken from atop a monkey kidney culture after a seven-day incubation period and enriched with the addition of 2% calf serum; d) regenerated fluid + 2% dialyzed calf serum; e) Hanks fluid with 0.3% glucose + 0.5% hydrolyte of lactoalbumin + 2% calf serum. The nutrient media were always prepared from the same reagents 4) Reagents for determining sugars by the Bertrand method.

B. Evaluation of the growth of tissues. Freshly isolated kidney tissue was macerated with a 0.25% solution of trypsin, and the cell emulsion obtained was transferred to Roux bottles. Into each bottle was put nine million cells and 150 milliliters /ml/ of nutrient medium. 24-hourly observations made of the growth (by microscopic method) with simultaneous determination of the changes in pH and glucose

consumption. The degree of growth of the tissue is shown in table 1. [tables and figures appended to article]

C. Measurement of Glucose Content. The sugar level was generally determined by the Bertrand method in samples of media from the top of the culture. The first determination, however, was made in the following manner: the cells, immediately after trypsinization of the kidney tissue, were computed and suspended in the nutrient medium. A sample of this emulsion was devirused, and the glucose content in the medium over the sediment was determined. This time was designated as zero, since the cells remained in the nutrient medium only during the preparatory activities. The next determinations were made every 24 hours for a total of 168 hours, the same volume of medium for samples always being taken from the top of the culture. In these samples the reducing substances were determined by the Bertrand method. The standard was 0.1% glucose solution, and all the nutrient media (without tissue) were used in the experiment. The samples of these media (controls) were determined simultaneously with the respective samples of those media under examination which contained cells. The nutrient medium was dealbuminized with $CuSO_4$ and, in accordance with the Bertrand method, by using the reducing properties of glucose, was titrated with a 0.1% solution of $KMnO_4$. From the number of mls of $KMnO_4$ used the glucose content remaining in the nutrient medium was computed.

D. In every sample, pH was determined with the aid of a pH-meter (radiometer).

E. Multiplication of the virus. The effect of the glucose content in the nutrient substrate on the multiplication of the poliomyelitis virus was studied ol known strains of Brunhild, MEF_1 and Saukett. The name of the multiplied virus was determined on the basis of the cytopathogenic effect in $TCID_{50}$/ml by the Reed and Muench method.

F. Chromatographic determinations. In order to verify the identity of the reducing substances determined with glucose, chromatographic determinations were made. Samples of the following substances were applied to a sheet of blotting paper: standard of 0.1% glucose solution, in the amount of 100 mcg, 50 mcg and 25 mcg, respectively; normal nutrient medium, i.e., with 0.1% glucose content, in which there were no cells; and the same medium, taken from the top of the culture at 24-hour intervals, from 0 to 168 hours. These samples were applied in the same quantities as the standard solution. Butanol with acetic acid was used as a solvent. The chromatogram, after being developed, was dried at room temperature, then at a temperature of 105° for five minutes.

Samples of media from which certain components had been eliminated -- such as serum and lactoalbumin in one, only the serum in the next, the Hanks salts in another (the remaining ones: lactoalbumin serum, sodium carbonate and phenol red) -- were placed on a separate sheet. As the standard -- a solution of 0.1% glucose.

RESULTS

On the basis of the results obtained it has been ascertained that the cells obtained from freshly isolated monkey kidney tissue grow and multiply in media with different glucose contents, the difference being only in the rate of growth.

The nutrient value of glucose in the period of weekly growth of the tissues studied is shown in Table II. No growth was observed in any of the media studied during the time-span from zero to 24 hours. Cell growth began earliest in the regenerated medium (after 48 hours), then in the 0.3% glucose fluid (after 72 hours). In the medium without glucose, and with dialyzed serum, growth commenced the latest (after 96 hours). After 120 hours the most abundant growth was observed in the regnerated media and in the medium with 0.3% glucose. After seven days' growth of the culture, microscopic observations (magnification 17 x 5) revealed that basically all of the media studied lent themselve to the cultivation of monkey kidney cells -- the only differences noted were those occurring during the start of cell growth, and changes in the morphology of the cells (Figs. 8 and 9).

The consumption of glucose by the monkey kidney cells in the period of their weekly growth is shown in Fig. 1. From the data compiled in this period it is evident that at zero time in all the varieties of media studied there was a considerable drop in the glucose content as compared with the initial quantity. The smallest drop was observed in Hanks fluid + 0.3% glucose. The glucose content dropped to 0.2%, or one-third the initial quantitys. Almost all the other media showed a two-thirds loss of glucose as compared with the initial quantity. In the period from 24 to 72 hours the greatest loss of glucose (or the greatest consumption) was recorded in all the media investigated; while after that period, down to 168 hours, this loss was more even and insignificant, and reached the same level regardless of the medium (with the exception of Hanks with 0.3% glucose). However, the glucose was not noted to have been consumed without some quantity of glucose remaining even after 240 hours of incubation of the cells.

As shown by Fig. 2, the pH of all the media studied changed in the course of cell multiplication -- from the starting time of the culture to 168 hours. These changes may be summarized by saying that down to 24 hours of cell incubation the pH of all the media rises considerably, from pH 6.9-7.0 to pH 7.7-7.8, after which there is noted an abrupt drop. From 48 hours hence, this drop is very slow in all the media studied. The greatest fluctuations were exhibited by the media with the highest glucose content: such as those with a 0.1% and 0.3% content; and media without glucose but with dialyzed serum, in which the pH at the starting time of the culture was 6.9-7.0, which rose to 7.7-7.8 in 24 hours, but which subsequently fell to 6.8-6.9 after a week's observation. The regenerated media, the pH of which was lower than that of the other media at the moment the culture was started, showed the least pH fluctuations: from pH 7.1-7.2 at the start to 7.4-7.8 after 24 hours, with a renewed decline within limits of 7.1-7.2 in the period down to 168 hours.

As shown in Figs. 3-7, the curves of the pH fluctuations and of glucose content stand in a certain relation to one another. The moment of abrupt drop of glucose content in the medium studied is, according to Fig. 2, preceeded by a drop in Ph, which begins about 24 hours earlier; in the period when the current consumption of glucose is small, a very slow drop in pH is likewise observed.

From the data compiled in Table II, it may be seen that the polio virus multiplied on the cultures incubated media with different glucose content (from 0.02 to 0.3%) shows no essential differences in the height of the "name" [sic]. It is possible that these differences would be considerably more pronounced if larger quantities of glucose were added to the nutrient medium.

Chromatographic identification of the reducing substances determined in the media studied without culture as compared with the glucose standard showed that the intensity of coloring of the spots was proportional to the quantity of the sample applied (that is, 100 mcg of standard glucose had the same coloring as 100 mcg of the initial medium without culture). But the intensity of the coloring of the spots in the medium at individual hours of incubation became weaker and weaker as the cells grew. The spots obtained in the chromatogram from the media studied at different hours of growth of the culture did not, however, possess the same Rf as the standard glucose, which might give rise to doubt as to whether the reducing substances determined by the Bertrand method in the media from the top of the cell culture are

really glucose. But the elimination of individual components from the medium led to the conclusion that the mineral salts were the ballast hindering the glucose in the medium observed from attaining the same Rf as the standard glucose. In view of this, the results of chromatographic investigations were recognized as confirmation of the results obtained by the Bertrand method, and the reducing substances determined in the samples of media from the top of the culture as identical with glucose.

DISCUSSION

The fact that we obtained the growth of cells of the kidney of the Macaca irus and Macaca mulata monkeys in a medium without glucose cannot be the basis for concluding that glucose is not a source of energy for the division and growth of cells. From the experiments conducted, it can only be concluded that glucose is not the only source of energy for monkey kidney cells. As may be seen from the results discussed above, these cells in the case of the absence of glucose can make use of the oxidation of the non-carbohydrate components contained in the nutrient medium (15, 18). These results may be compared with those obtained by Morgan and Kutsky.

Not all cells devoid of carbohydrates, however, have the same ability to make use of other sources of energy for their growth. For example, trypsinized MB III cells devoid of glucose in a nutrient solution degenerate in the course of 48 hours (5).

As may be seen from Fig. 1, the highest glucose consumption occurs in the initial period of development of monkey kidney cells, i.e., in the period down to 72 hours. These results agree with those obtained by Nelson, Spratt, Gothoskar, Raina and Ramakrishnan (12, 16, 17), who observed that the consumption of glucose by cultures differs in different periods of cell development. Probably the highest glucose consumption obtained in our work down to 72 hours is connected with the period of most intensive growth of the monkey kidney cells.

After 72 hours begins a period in which the glucose-consumption curve falls gently; probably after this time the kidney cells enter into a period of growth. In this period the glucose requirements of the cells are so small that the media from the top of the culture studied after complete development of the tissue in the time after 168 hours have shown a nearly constant glucose content within limits of 0.04-0.6%, and complete consumption of glucose was never noted. This phenomenon was repeated likewise in media from which an effort

was made to remove the source of glucose, as well as in media with a normal quantity of glucose (0.1%) and with an increased quantity of glucose (0.3%). In samples taken even after 240 hours of incubation, certain remnants of glucose were still discovered. The largest amount of glucose remained (after 168 hours) in the medium whose initial glucose content was 0.3% (Fig. 1). While 0.005% remained in the medium with 0.1% glucose, 0.15% remained in the medium with 0.3% glucose. This fact indicates that the culture draws from the nutrient medium only as much glucose as it requires for its vital processes; any excess of glucose remains unused.

Similar observations to those just described have been made by Willmer (21), Ebeling (11) and Demuth (8), who, upon using higher glucose concentrations, obtained even toxic action on the tissues. But others, such as Willson, Johnson and Brues, have drawn from their investigations the conclusion that the quantity of glucose used does not remain in direct, simple relation to the growth of the culture. Tissue cultures subjected to the action of colchicine in a concentration entirely inhibiting mitosis subsequently consumed normal quantities of glucose. Moreover, cultures growing in a fluid with a 500 mcg% glucose content consumed more of it than those simultaneously growing in a medium with 100 mcg%, but the investigations of a fixed (ultwalona) culture of cells in different periods of development of these cultures showed in both cases the same number of developing cells as the mitotic number.

The more rapid growth of cells in the regenerated media in which there was no glucose may be interpreted as due to the presence of stimulating substances contained in those media and resulting from the metabolism of the cells already developed (10, 9, 4). Moreover, the regenerated media were always more acidy at the starting time of the culture than were the media with 0.1% and 0.3% glucose, and it appears that actually a more acid medium favors a more rapid cell growth. From the data presented in the literature of the field it is evident that there is a correlation between glucose consumption and pH levels. The higher the glucose consumption, the greater the cell growth and the greater the drop in pH (15). These observations agree with the data obtained from our investigations, as may be seen in Figs. 3-7 and Table I. The fact, evident from the Figs. 1 and 2 of the 24-hour difference between the commencing acidification of the medium and the period of heavy glucose consumption is to a certain degree unambiguous, since there was no possibility of verifying when and to what extent the glucose absorbed at zero hour and slowly released (in the half of the quantity observed) during the first 48 hours had begun to join in the metabolic processes

of the cells. In any case, the fact of a simple dependence existing between glucose consumption by the cells and the pH of their medium appears to arouse no doubt.

The cell growth obtained by us on regenerated media (i.e., on media already used once) devoid of glucose was perhaps still another proof of the possibility of making use not merely of the energy of the glycolytic cycle. A similar conclusion has been drawn by Lipman from studies of measurements of glucose consumption by tissues in the course of their development in a Warburg apparatus. He conjectured that the glucose is not the sole, unique source of energy for in vitro growth of tissue.

The data obtained from our experiments on the growth of monkey kidney cells in regenerated media without glucose have confirmed, and even enriched, the results of research done by Chanina et al. (7). These authors, ascribing to glucose the greatest significance for tissue growth, used media from the top of a seven-day culture for newly trypsinized cells. To this medium they also added 0.1% glucose, in contrast to the procedure followed in our research. Their results, with respect both to tissue development and to the multiplication of poliomyelitis virus, were very close to those obtained with the use of freshly-prepared media. On the basis of these data, the authors concluded that glucose is the component most intensively consumed by cells cultivated in vitro. Our studies, on the other hand, have shown that the cells in the regenerated media can grow without glucose.

Still unexplained is the constantly recurring phenomenon that immediately after starting the culture -- that is to say that at zero hour, there occurred in all the media a considerable drop in the content of glucose as compared with the quantity of glucose in the initial medium. This heavy absorption of glucose takes place in the period when the incipient vital processes of the cells do not require such considerably expenditures of energy. The attempts thus far made to clarify this phenomenon are not satisfactory. However, the trend of further considerations admits the possibility (fakt) of a partial release of the sugar absorbed during the first 24 hours.

CONCLUSIONS

1. Kidney cells from monkeys of the species Macaca Mulata and Macaca irus have the ability to grow in medium with a 0.02% glucose content as well as in media without glucose, the only difference being in the rate of growth and in the morphology of the cells.

2. The monkey kidney cells multiply in the nutrient medium without glucose and with dialyzed serum. The rate of growth is, however, considerably slower than in all the other media studied. The morphology of the cells growing in this medium show certain changes.

3. The growth of cells in regenerated media, both with full serum and with dialyzed serum, ensued most rapidly.

4. The highest glucose consumption is observed in the initial phase of growth, i.e., during the first 72 hours of cell incubation.

5. Regenerated media may, in starting a fresh culture, be substituted for fresh media, after adding 2% calf serum to them.

6. The pH level remains in a state of simple dependence upon glucose consumption.

7. The name (miano) of the poliomyelitis virus multiplied on the cultures incubated in the several media studied show no essential differences.

The authors express their thanks to E. Janczurcza for his valuable suggestions and his assistance in executing chromatographic determinations.

SUMMARY

The influence of glucose on the development of non-passed tissue culture from monkey kidney (rhesus and cynomolgus species) has been studied. Nutrient media without glucose and regenerated media were used for growing the above mentioned tissue culture.

The results obtained show that the tripsinised tissue multiplicate in all the media, but there are differencies in the velocity of growth and in morphology of cells.

The growth appears most quickly in regenerated media.

The growth in media containing 0.1 and 0.3 percent of glucose appears later, after 48-72 hours, in those media the growth begins simultanously, but in the medium containing 0.3 percent of glucose it is quicker.

The growth in medium without glucose occurs latestly.

After 7 days the cells form monolayer in all kinds of media.

The cells utilize most of glucose during the period of the highest growth it means during the first 72 hours then the consumption of glucose decreases.

Some amount of glucose is not consumed even after 240 hours.

There are differents in morphology of cells dependent on the kind of medium. The cells in medium containing 0.1 percent glucose are long and have thick walls. The cells in medium without glucose are longer than in medium containing 0.1 percent of glucose and have thin walls. The cells in medium containing increased amounts of glucose are short with thick walls.

The titer of poliomyelitis virus grown on the investigated media was slightly higher in the medium containing 0.3 percent of glucose.

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Table I

Growth of Monkey Kidney Cells in Media with Different Glucose Content

a) Rodzaj płynu	b) 24 godz.	48 godz.	72 godz.	96 godz.	120 godz.	144 godz.	168 godz.
1) Hanks + 0,1% glukozy	–	–	+	++	+++	++++	++++
2) Hanks + 0,3% glukozy	–	–	++	+++	++++	++++	++++
3) Regen. + 2% sur. norm.	–	+	+++	++++	++++	++++	++++
4) Regen. + 2% sur. dial.	–	+	+++	++++	++++	++++	++++
5) Hanks bez gluk. + 2% sur. dial.	–	–	–	+	++	+++	++++

–) absence of growth; +) growth of individual cells; ++) incipient zone of growth; +++) pronounced zone of growth; ++++) whole surface covered with a solid layer of cells.

[Legend:] a) Kind of fluid; b) hours; 1) Hanks + 0.1% glucose; 2) Hanks + 0.3% glucose; 3) Regen. + 2% normal serum; 4) Regen. + 2% dialyzed serum; 5) Hanks without glucose + 2% dialyzed serum.
"Regen." = regenerated medium

Table II

Name of Poliomyelitis Virus Multiplied on Media with Different Glucose Content

a) Nr hodowli	b) Hanks+0.1% glukozy +2% sur. cielęcej	c) Hanks bez glukozy + 2% sur. dializ.	d) Płyn regen. + 2% surowicy	e) Płyn regen. + 2% surowicy dializ.	f) Hanks+0.3% glukozy
24	6,8	6,8	6,66	6,66	6,96
26	7,96	7,96	7,26	7,96	8,06
28	6,0	6,66	6,86	6,70	7,15
30	6,55 6,0	6,55 —	6,5 6,46	6,5 —	6,95 —
32	6,65 6,75	6,46 6,86	6,46 6,95	6,66 —	6,35 —
g) Średnia	6,66	6,81	6,58	6,93	7,09

[Legend:] a) Number of culture; b) Hanks + 0.1% glucose + 2% calf serum; c) Hanks without glucose + 2% dialyzed serum; d) Regen. fluid + 2% serum; e) Regen. fluid + 2% dialyzed serum; f) Hanks + 0.3% glucose; g) Average.

Fig. 1

Glucose Content in %

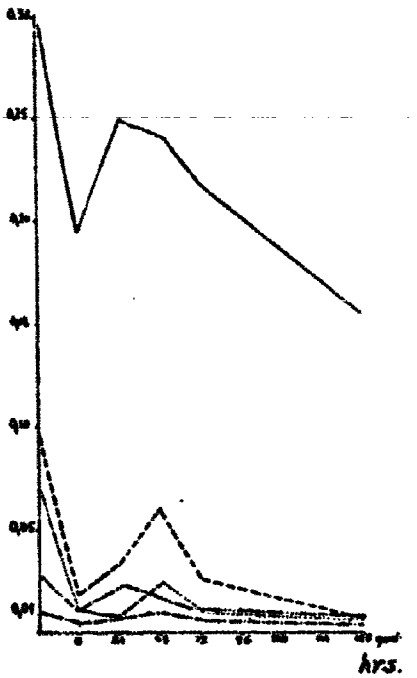
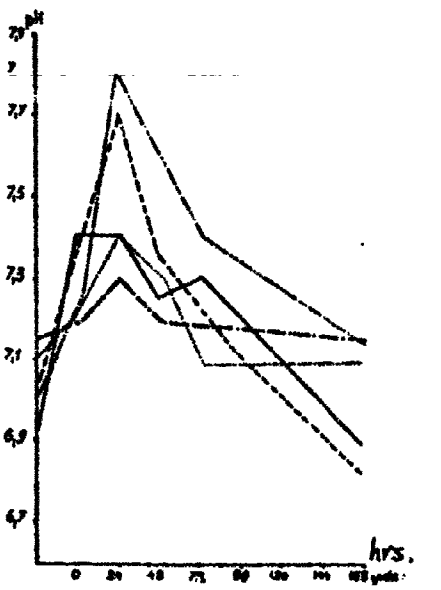


Fig. 2

pH Level with Different Glucose Content



---------- normal fluid - Hanks fluid + 0.1% glucose

———————— Hanks fluid + 0.3% glucose

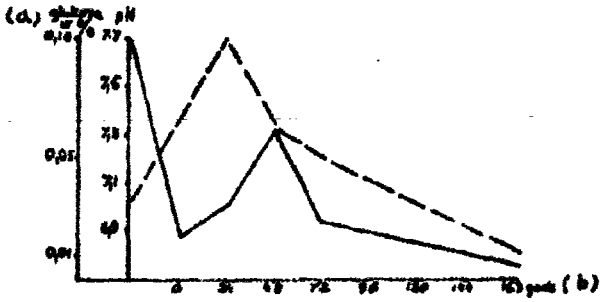
. . . . . regenerated medium + normal serum

– – – – – – regenerated medium + dialyzed serum

– – – – – Hanks fluid without glucose + dialyzed serum

Fig. 3

Glucose Consumption and pH of the Medium
(normal fluid)



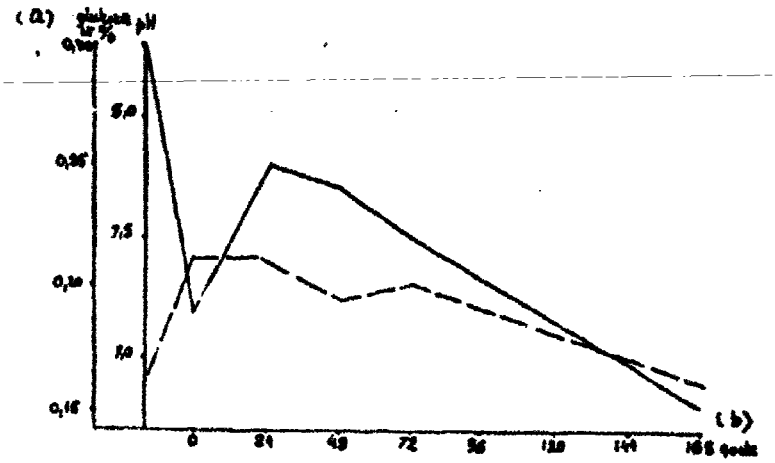
——————— glucose

– – – – – – pH

[Legend:] a) glucose in %;
b) hours.

Fig. 4

Glucose Consumption and pH of the Medium
(Hanks fluid + 0.3% glucose)



——————— glucose

– – – – – – pH

[Legend:] a) glucose in %;
b) hours.

Fig. 5

Glucose Consumption and pH of the Medium
(regenerated fluid + normal serum)

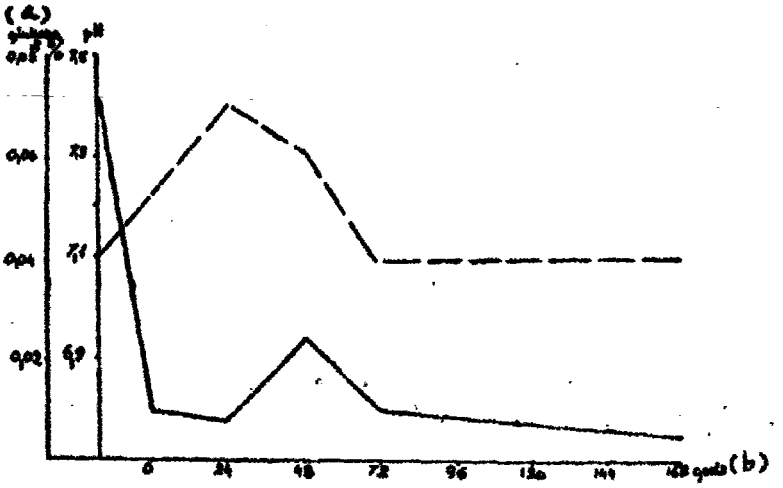
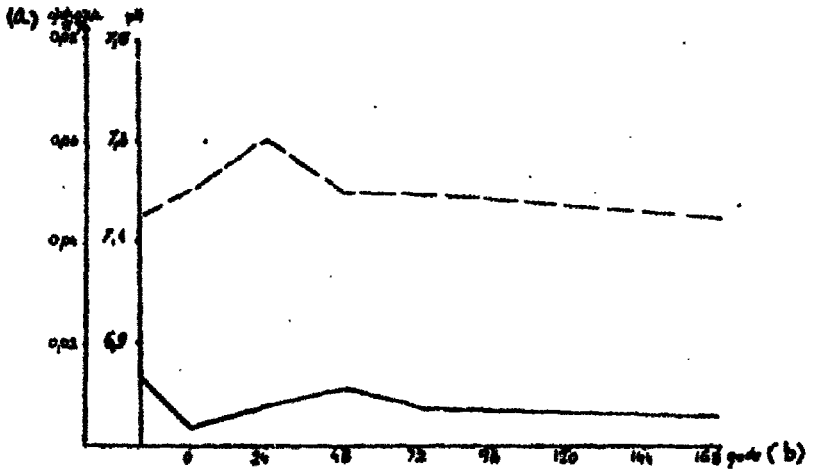


Fig. 6

Glucose Consumption and pH of the Medium
(regenerated fluid + dialyzed serum)

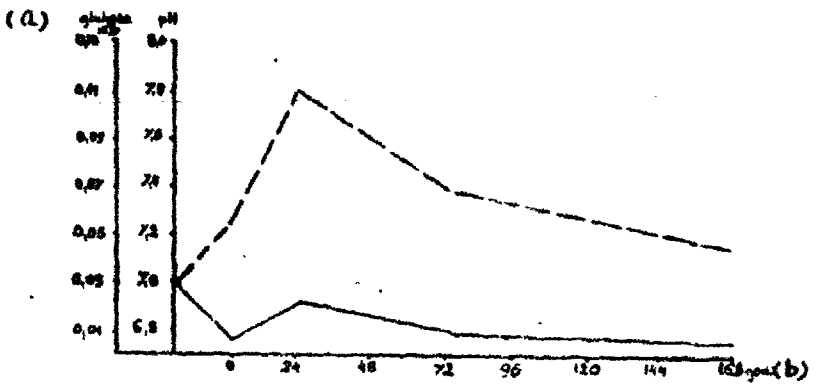


glucose
pH

[Legend:] a) glucose in %;
b) hours.

Fig. 7

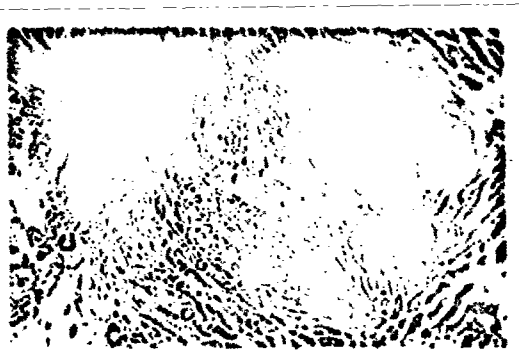
Glucose Consumption and pH of the Medium (Hanks fluid without glucose + dialyzed serum)



——— glucose
– – – – – – pH

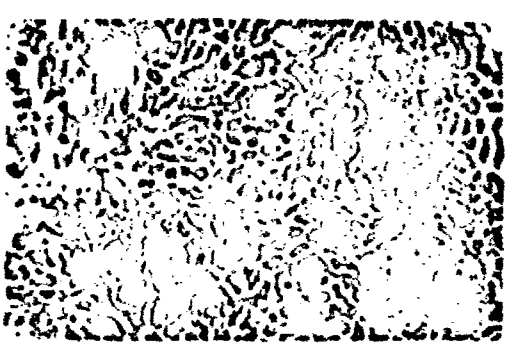
/Legend:/ a) glucose in %;
b) hours.

Fig. 8



Tissue culture in Hanks fluid with dialyzed serum without glucose.

Fig. 9



Tissue culture in Hanks fluid with normal serum + 0.3% glucose.

- END -